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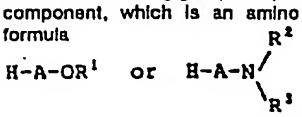
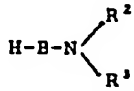
(54) A process for enzymatic production of dipeptides.

(57) Dipeptides having the general formula
H-A-B-Y
wherein A represents an optionally side-chain protected L- or D-α-amino acid or ω-amino acid and B represents an optionally side-chain protected L- or D-α-aminocarboxylic acid which may be the same as or different from A, an L- or D-aminophosphonic acid or L- or D-aminosulfonic acid or the corresponding ω-amino acids or salts and hydrates thereof, and Y is OH or a C-terminal blocking group, are prepared by reacting a substrate component, which is an amino acid derivative having the formula



wherein A is as defined above, R^1 represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents or an α-des-amino fragment of an amino acid, and R^2 and R^3 are the same or different and each represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents,
with a nucleophile component which when A – B may be formed in situ and is selected from (a) L-amino acids having the formula
H-B-OH
(b) L-amino acid amides having the formula



wherein B is an L-amino acid, and R^2 and R^3 have the above meaning, except that when R^2 represents hydrogen, R^3 may also represent hydroxy or amino
(c) L-amino acid esters having the formula
H-B-OR^4
wherein B is an L-amino acid, and R^4 represents alkyl, aryl or aralkyl, and
(d) optionally acid group protected straight chain or branched amino phosphonic acids or amino sulfonic acids having the formula
$NH_2C_xH_zPO_3H_2$ or $NH_2C_xH_zSO_3H$
wherein x is 1-6 and z is 2-12
in the presence of a serine or thiol carboxypeptidase from yeast or of animal, vegetable or other microbial origin, preferably CPD-Y from yeast in an aqueous solution or suspension having a pH value between 5 and 10.5 optionally containing an organic solvent and/or a salt, and then, if desired, cleaving a present side-chain protecting group or protective group Y and/or, if desired, converting the resulting dipeptide derivative to a salt or hydrate.

The process allows for production of L,L-, LD-, DL- and DD-dipeptides without risk of racemization in a simple and economic manner.

EP 0 278 787 A1

Description

A process for enzymatic production of dipeptides

The present invention concerns a process for enzymatic production of dipeptides and derivatives of dipeptides and of the type stated in the introductory portion of claim 1.

In recent years there has been an increasing interest in dipeptides and dipeptide derivatives optionally containing an amino-acid residue of D-configuration, with a view to their potential pharmacological effects, such as e.g. antibiotics. Likewise, there has been a great interest in dipeptides within fields such as artificial nutrition - human as well as veterinary, sweeteners and within agrochemistry, such as e.g. herbicides.

Such dipeptides H-A-B-Y can be produced by means of known chemical coupling reactions, but all these methods share the feature that; generally, it is necessary to protect the amino acids involved - A and B - on the amino group and the carboxylic acid group, respectively, and frequently also on the side chains if these carry functional groups. Further, there is an inherent risk of side reactions during the chemical coupling step because of the reagents and conditions employed, a major side reaction being racemization, particularly of the A-component. By replacing the chemical coupling step with an enzymatic coupling step proceeding under mild conditions, such side reactions and racemization can be avoided, yielding a stereochemically pure product.

The presence of amino- and carboxyl protective groups is mandatory in chemical coupling as well as in enzymatic coupling using endoproteases, and according to prior knowledge also on the amino function of the substrate in enzymatic exoprotease catalyzed formation of dipeptides.

This adds several undesired features to these processes seriously afflicting their process economy on an industrial scale, particularly apparent in dipeptide synthesis.

The disadvantages are concerned with the introduction of these groups, as well as their removal and presence during process operation, increasing overall process cost and time and affecting overall yield.

Typical examples of amino protective groups commonly used are those of the carbobenzoxy (Z-) and tertbutoxycarbonyl (Boc-) type, which are of a molecular weight comparable to those of the amino acid residues. Firstly, the protective groups will have to be introduced in the starting materials by means of appropriate costly agents in a separate reaction step followed by an isolation step. While present, these hydrophobic groups often have a drastical effect upon the solubility of the intermediates and reaction products, and may afflict both the nature and the amount of solvents required in their processing as well as ease of purification and of deprotection. The deprotection will also take place in a separate step with a following purification step.

For this purpose a series of reactions are available, but with the exception of catalytical hydrogenation, posing industrial problems of its own, these methods are occuring under violent often strongly acidic or basic conditions, frequently giving rise to a series of side reactions, resulting in an impure product of demanding laborious purification.

The last steps in this relatively long series of synthesis steps may thus be a rather comprehensive deprotection to obtain the desired peptides, and, owing to the almost inevitable secondary reactions, rather laborious purification procedures are frequently required to provide a product with the desired high purity.

Attempts to avoid amino terminal protection in the production of dipeptides have led to microbial fermentation approaches, like the fermentation process under formation of aspartame described in EP-A1-074095 and EP-A2-102529. This technique is fundamentally different from synthetic approaches and relies on specific organisms for each peptide, and is thus not generally applicable. In addition, yields are often low and recovery from the fermentation broth laborious.

Thus, it is an obvious advantage in terms of overall process economy to be able to avoid protective groups, also on the amino and carboxy termini. It is the object of the present invention to make this possible in the synthesis of dipeptides mediated by serine- and thiol carboxypeptidase catalysis. In some cases, it may be of interest to be able to produce a dipeptide which may carry side-chain protection, but no terminal protection, and it will be shown that it is possible in the process according to this invention, starting from side-chain protected, but amino respectively carboxy unprotected starting materials. In this case, the same advantages of mild reaction conditions and overall process economy may be obtained. If desired, the side-chain protective group may be cleaved by chemical or enzymatic means.

The enzyme catalyzed coupling reactions enabling the use of side-chain unprotected amino acid derivatives and an optionally C-terminal unprotected B-component (nucleophile) are known. See e.g. EP-A-0017485 or the granted equivalent (EP-B1-17485).

EP-B1-17485 describes a process for producing peptides of the general formula

A_1-B_1-Z_1

wherein A_1 represents an N-terminal protected amino acid residue or an optionally N-terminal protected peptide residue having a C-terminal L-amino-acid residue, and B_1 represents an L-amino acid residue, and Z_1 is OH or a C-terminal protective group, by reaction of the substrate component with an amine component in the presence of an enzyme, and, if desired, cleavage of optional terminal protective groups to provide a peptide of the formula

A_1-B_1-Z_1

which is characterized by reacting a substrate component selected from

amino-acid esters, peptide esters, depsipeptides, or optionally N-substituted amino acid amides or peptide amides, or optionally N-terminal protected peptides of the formulae

A_1-OR_1, A_1-NR_2R_2' or A_1-X_1-OH

wherein A_1 is as defined above,
R_1 is alkyl, aryl, heteroaryl, aralkyl or an α-desamino fragment of an amino acid residue,
R_2 and R_2' are each hydrogen, alkyl, aryl, heteroaryl or aralkyl, and
X_1 is an L-amino-acid residue,
with an amine component (nucleophile) selected from optionally N-substituted L-amino-acid amides, L-amino acids or L-amino-acid esters, of the formula

H-B_1-NR_3R_3' or H-B_1-OR_4

wherein B_1 is as defined above,
R_3 and R_3' are each hydrogen, hydroxy, amino, alkyl, aryl, heteroaryl or aralkyl, and
R_4 is hydrogen, alkyl, aryl, heteroaryl or aralkyl,
in the presence of an L-specific serine or thiol carboxypeptidase enzyme originating from yeast or of animal, vegetable or other microbial origin, in an aqueous solution or dispersion at pH-5-10.5, preferably at a temperature of 20-50°C. The preferred enzyme is carboxypeptidase Y from yeast, called CPD-Y in the following. (It is noted that in order to avoid confusion with the present symbol meaning the above-mentioned symbol meanings are not identical with those used in EP-B-17485).

Thus, if a dipeptide is to be produced by the process of EP-B1-17485, the substrate component is obligatory an N-terminal protected amino-acid derivative, and the constituent amino acid is obligatory an L-amino-acid.

More generally, it is said that the need for N-terminal amino group protection of the substrate component decreases with increasing chain length of the peptide residue and is substantially absent when the peptide residue consists of 3 amino-acids, depending, however, upon their type and sequence.

This is illustrated by Breddam et al, "Influence of the substrate structure on carboxypeptidase Y catalyzed peptide bond formation", Carlsberg Res. Commun., vol. 45, p. 361-67, 30th December 1980, in which Ac-Ala-Ala-Ala-OMe and H-Ala-Ala-Ala-OMe were coupled with H-Leu-NH_2 in the presence of CPD-Y to form Ac-Ala-Ala-Ala-LeuNH_2 and H-Ala-Ala-Ala-LeuNH_2 in yields of 90 and 80%, respectively.

Breddam et al also examined the importance of the amino acid configuration for the coupling yield in CPD-Y catalyzed peptide synthesis, cf. the following table, where Ala represents L-alanine and ala represents D-alanine.

Substrate	Product	Yield (%)
H-Ala-Ala-Ala-OMe	H-Ala-Ala-Ala-Leu-NH_2	80
H-Ala-ala-Ala-OMe	H-Ala-ala-Ala-Leu-NH_2	40
H-Ala-Ala-ala-OMe	H-Ala-Ala-ala-Leu-NH_2	0

Conditions: 25 mM substrate, 0.1 M KCl, 1 mM EDTA, pH 9.5, CPD-Y = 12 µm, 0.2 M Leu-NH_2, The reaction was stopped after 20 min.

It appears from the table that if the C-terminal is in D-form, like in H-Ala-Ala-ala-OMe, no peptide synthesis takes place because the ester is not reacted. With the D-amino acid juxtaposed the C-terminal like in H-Ala-ala-Ala-OMe, reaction takes place, but the coupling yield is reduced to 40% compared with the 80% in the pure L-configuration H-Ala-Ala-Ala-OMe.

Further, it has long been known that some endoproteases can catalyze oligomerization of certain N-unprotected amino acid esters with L-configuration, but it has never been attempted to use this for production of dipeptides which are not simple dimers. Generally, the results of such observations have been a mixture of a series of oligomers, sometimes long, and only in case of product precipitation has it been possible to isolate a single product.

For this reason the use of endoproteases for peptide synthesis has been limited to the use of amino and carboxy terminal proteced starting materials, as exemplified by US-A-4 086 136.

These types of starting materials are also mandatory if aspartate endoproteases are used as exemplified by US-A-3 972 773, and if metallo-endoproteases are used, as exemplified by the synthesis of Z-AspPhe-OMe.PheOMe-salt in EP-A1-009585.

Finally, the synthesis of the diastereomeric dipeptides of DL, LD and DD-configuration as well as peptides containing beta-amino acid residues from amino-unprotected starting compounds has so far not been

possible with carboxypeptidases nor in general with any proteolytical enzymes (Class EC 3.4). Some efforts have been made with a different class of enzymes, aminoacyl-t-RNA-synthetase (Class EC 6.1) as exemplified by EP-A1-086053. In this case a specific enzyme must be used for each type of amino acid residue, and furthermore, expensive Co-factors like ATP are required. At the same time, yields are very poor, so even though some product was isolated and identified, typically a ten-fold excess of Co-factor and a hundred fold excess of nucleophile and up to a thousand fold excess of enzyme by weight was required.

It has now surprisingly been found that the serine and thiol carboxypeptidases used in EP-B1-17485 are capable of utilizing N-unprotected amino-acid esters as a substrate component in controlled reactions for synthesis of dipeptides and dipeptide derivatives, and that it is possible to suppress a possible oligomerization of the substrate.

It has moreover surprisingly been found that also N-unprotected amino acid derivatives of D-configuration can be used as substrates in these reactions, so that, in addition to LL-dipeptides, it is also possible to synthesize DL-dipeptides. The reaction rate for D-substrates, however, is generally somewhat lower than for L-substrates under uniform conditions, but, as illustrated below, the difference in rate is much smaller than for the corresponding N-protected amino acid esters, the D-substrate being reacted at a rate which is much smaller than the rate for the L-substrate. The yields are often just as high or higher with the unprotected D-substrates in relation to the unprotected L-substrates as shown by the following examples.

Furthermore, it has even more surprisingly been found that with substrates of this structure, nucleophiles of D-configuration can be coupled by means of these enzymes, otherwise known to be L-specific on the amino side of the synthesis point. An amino acid ester incorporated in this manner is not hydrolyzed further.

The process of the invention is thus characterized by the features defined in the characterizing portion of claim 1.

Examples of useful amino-acids include aliphatic amino-acids, such as monoaminomonocarboxylic acids, e.g. glycine (Gly), alanine (Ala), valine (Val), norvaline (Nval), leucine (leu), isoleucine (iso-Leu) and norleucine (Nleu), hydroxy amino-acids, auch as serine (Ser), threonine (Thr) and homoserine (homo-Ser), sulfur-containing amino-acids, such as methionine (Met) or cystine (CysS) and cysteine (CysH), monoaminodicarboxylic acids, such as aspartic acid (Asp), glutamic acid (Glu) and amides thereof, such as asparagine (Asn) and glutamine (Gln), diaminomonocarboxylic acids, such as ornithine (Orn) and lysine (Lys), arginine (Arg), aromatic amino-acids, such as phenylalanine (Phe) and tyrosine (Tyr), as well as heterocyclic amino-acids, such as histidine (His), proline (Pro) and tryptophan (Trp). Examples of useful amino-acids of a more unusual structure are penicillamine (Pen), aminophosphonic acids, such as alanine-phosphonic acid (Alap), aminosulfonic acids, such as taurine (Tau), and ω-amino-acids, such as β-alanine (BAla). As mentioned, they may be included in D-form in the substrate component and they may also be present in D-form in the nucleophile component.

The advantages of the process of the invention over the given known methods are that minimum or no side-chain protection is required, that no N-protection of the substrate component (which may have both D- and L-configuration) is required, that there is little or no risk of racemization, that there are few synthesis steps, and that a relatively pure end product is obtained. The process therefore provides an extremely simple and economic method of production.

Preferred substrate components are esters in which R^1 is a straight or branched alkyl group having 1 to 6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, amyl and hexyl, or the aralkyl group benzyl. Particularly preferred nucleophile components are free L-amino-acids or amino-acid amides, in which R^2 is H and R^3 is H or C_1-C_6 alkyl, or amino-acid esters in which R^4 is a straight or branched alkyl group having 1 to 6 carbon atoms such as any of those given above. More generally, as defined above, R^1 may be alkyl, aryl or aralkyl optionally substituted by inert substituents, e.g. hydroxy or nitro. R^1 will usually have no more than 12 carbon atoms, and substituents usually no more than 6.

The invention also comprises the processes involving intermediate formation of a peptide containing the group $-NR^2R^3$ or $-OR^4$, following which this group is cleaved to form a carboxylic acid group. This cleavage may be catalyzed by another or the same enzyme as was used to form the peptide.

Enzymes may also be used to cleave side-chain protective groups. Suitable enzymes are proteolytic enzymes, lipases and esterases, according to the nature of the protective group, see "The Peptides, Analysis, Synthesis, Biology" Vol 9, Special Methods in Peptide Synthesis Part C, by J. A. Glaas, Enzymatic manipulation of Protecting Groups in Peptide Synthesis, Academic Press 1987.

The process of the invention may be performed with CPD-Y, which is the presently preferred enzyme, and which is characterised more fully in EP-B1-17485, and also with other serine or thiol carboxypeptidases, such as those mentioned in the survey below, since these have a common mechanism of activity via acyl enzyme intermediates.

Enzyme	Origin
	Fungi
Penicillocarboxypeptidase S-1	Penicillium janthinellum
- S-2	- -
Carboxypeptidase(s) from	Aspergillus saitoi
-	Aspergillus oryzae
	Plants
Carboxypeptidase(s) C	Orange leaves
	Orange peels
Carboxypeptidase C_N	Citrus natsudaidai
Hayata	
Phaseoline	Bean leaves
Carboxypeptidase(s) from	Sprouted barley or malt
	Sprouted cotton plants
	Tomatoes
	Watermelons
	Bromelein (pineapple)
	powder

The close relation between many of the above-mentioned carboxypeptidases has been discussed by Kubota et al, Carboxypeptidase C, J. Biochem., Vol. 74, No. (1973), p. 757-770. Carboxypeptidases from malt, wheat and other sources have been described by Breddam, Carlsberg Res. Comm. Vol. 51, p. 83-128, 1986.

The carboxypeptidase used may also be chemically modified or be a biosynthetic mutant of the natural form.

As illustrated more fully below, the process of the invention is rather simple; however, it is important to maintain a resonably or substantially constant pH value in the reaction mixture. This pH value is between 5 and 10.5, and preferably between 7 and 9.5, but also depends upon the particular starting materials, the peptide formed and the enzyme.

The reaction is performed in an aqueous reaction medium, if desired containing up to 70% of an organic solvent which is miscible or immiscible with water, and compatible with the enzyme under the conditions specified. Preferred solvents are lower alcohols, dimethylformamide, dimethyl sulfoxide, dimethoxyethane, ethylene glycol and ethyl acetate.

The reaction temperature is preferably room temperature or above, e.g. 20 to 50°C, but temperatures in the range 0 to 60°C may be used. The most advantageous temperature may be chosen according to the conditions otherwise given.

The concentration of the two reaction components may vary within wide limits, but the nucleophile component is frequently in excess. In order to avoid oligomerization of the substrate component, the nucleophile component is often added in small portions at intervals during the entire reaction sequence. If an ethyl or higher ester is used, surprisingly no oligomerization is observed, and very high substrate concentrations may be used without side-reactions. In this case, a homo-dipeptide in which A = B may be formed without oligomerization from a single starting component, the nucleophile being generated in situ by hydrolysis of this ester. In some cases, this is a further technical advantage, cf. Fig. 2.

Thus, the starting concentration of the substrate component may typically be 0.005 to 2 molar and for the nucleophile component, in cases where it is added separately, 0.005 to 3 molar. In most situations, it is possible to recover excess nucleophile component and the hydrolysis product from the substrate component for optional re-esterification and re-use. Recycling of the components is particularly easy because of their simple structure and the absence of side-reactions and deprotective losses.

The enzyme concentration may likewise vary, but is frequently somewhat higher (5-50 µm) than the concentrations appropriate for the use of N-protected amino-acid ester substrates. However, as illustrated by the following Examples, the amount required for synthetic purposes may be reduced more than ten-fold by using a stable immobilized enzyme preparation, thereby enabling the enzyme to be used in a continuous process.

The reaction medium may also contain salts, such as NaCl, which influence the binding of the enzyme to the

substrate, as well as a complexing agent, such as EDTA, for metal ions present, which stabilises the enzyme.

The reaction rate differences between D- and L-substrates mentioned above are illustrated in Fig. 1, which shows the reaction sequence for the CPD-Y hydrolysis of a protected and unprotected amino-acid ester (Tyr-OEt) in D- and L-form. It will be seen from the Figure that, while L-AcTyrOEt is hydrolyzed almost instantaneously, only insignificant hydrolysis (below 5%) of D-AcTyrOEt has taken place after 2 hours.

In contrast, thre are only small differences in the hydrolysis sequence for the unprotected esters in L- and D-form.

This surprising hydrolysis sequence is reflected in the following Examples, which illustrate the production of various dipeptides in the process of the invention using the enzymes CPD-Y and malt carboxypeptidase II, (CPD-MII) and wheat carboxypeptidase (CPA-W).

General method for Examples 1-15

The reactions, performed on an analytical scale with a reaction volume of 1 ml, were carried out in a pH-stat, and the selected pH value was kept constant by automatic addition of 1 N NaOH. Reaction temperature was room temperature, unless otherwise stated. The table also includes reaction concentrations, content of organic solvent, product and yield. Reaction times are typically between 0.5 and 5 hours, and enzyme concentrations are typically 10-20 µm, unless otherwise stated.

Product identification and determination of product yield were performed by means of reverse phase HPLC (Waters 6000 A pumps, 660 gradient blender, UK 6 injector) on a C_{18}NOVA PAK column (Waters, RCM) using suitable gradients of elution systems containing 50 mM triethylammonium phosphate, pH 3.0 from 0% to 80% acetonitrile with a flow of 2 ml/min. Elution was monitored by means of a UV detector (Waters 480) at 230 nm, 254 nm, 278 nm or 290 nm.

The products were identified by amino-acid analysis of fractions from the HPLC analysis, which corresponded to the assumed product peak and/or by HPLC comparison with a chemically-synthesized reference product. These were produced according to known principles, usually via reaction between BOC-A-OSu - tertiary-butyloxycarbonyl - the succine imide ester derivative of the substrate amino-acid - and the used nucleophile component followed by deblocking of the N-terminal amino acid residue. In all cases, it was possible to separate LL-and DD-dipeptides from the diasteromeric DL-and LD-dipeptide products.

For the products which can only be detected at 230 nm, the product yields were determined by means of the absorption/concentration curve of the chemically synthesized reference compound. For the other products, the yields were determined on the basis of the ratio between the integrated areas below the peaks in the elution chromatogram, corresponding to product respectively the reactant which absorbs at the wavelength concerned.

The reaction conditions in the preparative Examples 16-21 are described in the individual Examples. The reactions were followed on analytical HPLC as described. The enzyme concentrations are generally lower and the reaction times longer than in the corresponding analytical Examples, but no attempt to optimize the reaction conditions has been made.

Example 1

Carboxypeptidase Y [a]) catalyzed synthesis of L-L-dipeptides with L-Tyrosine ethylester (50 mM) as substrate component and free amino-acids as nucleophiles

Nucleophile (conc.)		Solvent	pH	Product	Yield %
Alanine	(1.9 M)	Water	9.5	TyrAlaOH	10
Arginine	(0.8 M)	Water	9.5	TyrArgOH	31
Cysteine	(1 M)	Water	8.0	TyrCysOH	30
LD-Cysteine	(2 M)	Water	8.0	TyrCysOH(LL)	40
Leucine	(0.2 M)	Water	8.0	TyrLeuOH	1
Lysine	(2 M)	Water	9.5	TyrLysOH	18
Methionine	(0.3 M)	Water	8.0	TyrMetOH	8
Methionine	(0.3 M)	Water	9.0	TyrMetOH	9
Methionine	(0.3 M)	30% DMSO	9.0	TyrMetOH	15
Methionine	(0.3 M)	15% EtOH	9.0	TyrMetOH	7
Glutamine	(0.8 M)	Water	9.5	TyrGlnOH	8
Pennicil-amine	(0.5 M)	Water	8.0	TyrPenOH	7

[a]) 10 µM, 1 mM EDTA

Example 2

Carboxypeptidase Y [a]) catalyzed synthesis of L-L-dipeptides with L-Methionine (0.3 M) as nucleophile component in water at pH 9.0

Substrate (50 mM)	Product	Yield %
Leucine methylester	LeuMetOH	30
Leucine isopropylester	LeuMetOH	36
Methionine ethylester[b])	MetMetOH	25
Phenylalanine methylester	PheMetOH	16
Phenylalanine ethylester	PheMetOH	19
Phenylalanine isopropylester	PheMetOH	23
Serine isopropylester[c])	SerMetOH	21
Tryptophane methylester	TrpMetOH	26
Tyrosine benzylester[d])	TyrMetOH	19

a) 10 µM, 1 mM EDTA

b) 5 mM

c) Reaction time > 20 hr

d) 30% DMSO

Example 3

Carboxypeptidase Y [a] catalyzed synthesis of L-L-dipeptides with L-Tyrosine ethylester (50 mM) as substrate component and L-amino-acid amides or esters as nucleophiles

Substrate	Nucleophile	(Conc.)	Solvent	pH	Product	Yield%
TyrOEt	Leucine amide	(0.2 M)	30% DMSO	9.5	$TyrLeuNH_2$ [b]	42
					TyrLeuOH	4
TyrOEt	Lysine amide	(0.3 M)	Water	9.5	$TyrLysNH_2$	20
					TyrLysOH	22
TyrOEt	Arginine amide	(0.2 M)	Water	9.0	$TyrArgNH_2$	50
TyrOEt	Valine amide	(0.3 M)	Water	9.0	$TyrValNH_2$	77
TyrOEt	Leucine methylester	(0.2 M)	30% DMSO	9.0	TyrLeuOH	6

[a] 20 µM, 1 mM EDTA

[b] Reaction time > 20 hr, 50% substrate converted

Example 4

Carboxypeptidase Y [a]) catalyzed synthesis of L-L-homo-dipeptides from a single starting compound in water at pH 8.5

Substrate	(conc.)	Product	Yield %
Methionine methylester	(0.5 M)[b][c]	MetMetOH	14
Methionine ethylester	(0.5 M)	MetMetOH	16
Methionine isopropylester	(0.5 M)	MetMetOH	14
Tyrosine methylester	(0.2 M)	TyrTyrOH [c]	1
Tyrosine ethylester	(0.2 M)	TyrTyrOH [c]	1
Phenylalanine ethylester	(0.2 M)[d][e]	PhePheOH	2
Alanine amide	(0.2 M)[f][d]	$AlaAlaNH_2$	b)

a) 10 µM CPD-Y, 1 mM EDTA
b) Polymerization
c) Precipitation
d) pH 9.0
e) 30% DMSO
f) 50 uM CPD-Y, 1 mM EDTA

Example 5

Carboxypeptidase Y [a] catalyzed synthesis of D,L-dipeptides with D-Tyrosine ethylester (50 mM) as substrate and free L-amino-acids as nucleophiles in water

Nucleophile	(conc.)	pH	Product	Yield %
Arginine	(1 M)	9.0	tyrArgOH	75
Cysteine	(1 M)	8.0	tyrCysOH	86
LD-cysteine	(2 M)	8.0	tyrCysOH(DL)	45
Leucine	(0.2 M)	8.0	tyrLeuOH	22
Methionine	(1 M)	9.0	tyrMetOH	65
Pennicil-amine	(1 M)[b]	8.0	tyrPenOH	27

[a] 10 µM, 1 mM EDTA

[b] Reaction time > 20 hr

Example 6

Carboxypeptidase Y [a]) catalyzed synthesis of D,L-dipeptides with L-Methionine (0.3 M) as nucleophile component in water at pH 9.0

D-substrate (50 mM)	Product	Yield %
leucine methylester	leuMetOH	50
leucine isopropylester	leuMetOH	71
methionine ethylester	metMetOH	68
phenylalanine ethylester	pheMetOH	71
phenylalanine isopropylester	pheMetOH	72
serine isopropylester	serMetOH [b])	46
tryptophane ethylester	trpMetOH	72

[a]) 15 µM, 1 mM EDTA

[b]) Reaction time 5 days

Example 7

Carboxypeptidase Y [a]) catalyzed synthesis of D,L-dipeptide amides with D-Tyrosine or D-Phenylalanine ethylester (50 mM) as substrate component and L-amino-acid amides as nucleophile components in water

Substrate	Nucleophile	(conc.)	pH	Product	Yield %
tyrOEt	Leucine amide	(0.2 M)	9.0	$tyrLeuNH_2$	82
				tyrLeuOH	2
tyrOEt	Valine amide	(0.3 M)	9.0	$tyrValNH_2$	94
tyrOEt	Arginine amide	(0.2 M)	9.0	$tyrArgNH_2$	86
pheOEt	Alanine amide	(0.8 M)	9.0	$pheAlaNH_2$ [b])	50

[a]) 15 µM, 1 mM EDTA

[b]) Some polymerization noted

Example 8

Carboxypeptidase Y [a]) catalyzed synthesis of D,D-homodipeptide esters from D-substrate ester components in water at pH 9.0, also acting as nucleophile component

D-substrate	(conc.)	Product	Yield %
tyrosin ethylester	(0.05 M)	tyrtyrOEt	9
phenylalanine ethylester	(0.1 M)	phepheOEt	10
tyrosine ethylene glycolester	(0.05 M)	tyrtyrOEtOH	1
methionine methylester	(0.1 M)	metmetOMe	3

[a]) 15 µM, 1 mM EDTA

Example 9

Carboxypeptidase Y [a] catalyzed synthesis of L-L-dipeptides with side-chain-protected carboxy-terminal amino-acids with L-TyrOEt (50 mM) as substrate component and side-chain-protected L-amino-acids and amides as Nucleophiles

Nucleophile	(Conc.)	pH	Solvent	Product	Yield%
Acetamidomethyl cysteine	(1 M)	8.5	Water	TyrCys(-SAcm)OH	10
Acetamidomethyl cysteine amide	(0.4 M)	8.5	Water	TyrCys(-SAcm)NH_2	12
				TyrCys(-SAcm)OH	1
Beta-Benzyl-aspartic Acid	(0.1 M)[b]	9.0	30%DMSO	TyrAsp(OBzl)OH	13
Epsilon Trifluoracetyl-lysine	(0.1 M)[b]	8.5	30%DMSO	TyrLys(Tfa)OH	12
Gamma-Tert-butyl-glutamic Acid Amide	(0.1 M)[b]	8.0	30%DMSO	TyrGlu(OtBu)NH_2	3
				TyrGlu(OtBu)OH	12
Gamma-Methyl-glutamic Acid	(0.3 M)	8.5	Water	TyrGlu(OMe)OH	20
Gamma-Ethyl-glutamic Acid	(0.3 M)	8.5	Water	TyrGlu(OEt)OH	18

[a] 10 µM, 1 mM EDTA

[b] 25 mM Substrate, 20 µm

Example 10

Carboxypeptidase Y [a] catalyzed synthesis of D-L-dipeptides with side-chain-protected carboxy-terminal amino-acids with D-TyrOEt (50 mM) as substrate component and side-chain-protected L-amino-acids and amides as Nucleophiles

Nucleophile	(Conc.)	pH	Solvent	Product	Yield%
Acetamidomethyl-cysteine	(1 M)	8.5	Water	tyrCys(-SAcm)OH	75
Acetamidomethyl-cysteine amide	(0.4 M)	8.5	Water	tyrCys(-SAcm)NH_2	71
				tyrCys(-SAcm)OH	6
Beta-Benzyl-aspartic Acid	(0.1 M)[b]	9.0	30%DMSO	tyrAsp(OBzl)OH	54
Epsilon-Trifluoroacetyl-Lysine	(0.1 M)[b]	8.5	30%DMSO	tyrLys(Tfa)OH	51
Gamma-Tert-butyl-glutamic Acid Amide	(0.1 M)[b]	8.0	30%DMSO	tyrGlu(OtBu)NH_2	42
				tyrGlu(OtBu)OH	10
Gamma-Methyl-glutamic Acid	(0.3 M)	8.5	Water	tyrGlu(OMe)OH	75
Gamma-Ethyl-glutamic Acid	(0.3 M)	8.5	Water	tyrGlu(OEt)OH	71

[a] 10 µM, 1 mM EDTA

[b] 25 mM Substrate, 20 µm

Example 11

Carboxypeptidase Y [a]) catalyzed synthesis of L,L-dipeptides with side-chain-protected amino-terminal amino-acid residues from side-chain-protected substrate components (25 mM) and L-Methionine (0.3 M) as nucleophile component in 30% DMSO at pH 9.0

Substrate	Product	Yield %
L-Aspartic Acid Dibenzyl ester [b])	Asp(OBzl)MetOH	65
L-Glutamic Acid Dibenzyl ester [c])	Glu(OBzl)MetOH	70

[a]) 20 µM, 1 mM EDTA, Reaction time > 20 h
[b]) At 35 per cent conversion
[c]) At 60 per cent conversion

Example 12

Carboxypeptidase Y [a]) catalyzed synthesis of dipeptide esters and amides containing omega-amino-acids from L- and D-amino-acid ester substrates at 50 mM in water with beta-alanine and beta-alanine amide as nucleophiles

Substrate	Nucleophile	(conc.)	pH	Product	Yield %
tyrOEt	B-Alanine methyl ester	(0.2 M)	8.5	tyrBAlaOMe	51
tyrOEt	B-Alanine methyl ester	(0.5 M)	8.5	tyrBAlaOMe	72
pheOEt	B-Alanine methyl ester	(0.5 M)	9.0	pheBAlaOMe	83
PheOEt	B-Alanine methyl ester	(0.5 M)	9.0	PheBAlaOMe	15
pheOEt	B-Alanine amide	(0.5 M)	9.0	pheBAlaNH$_2$	70
PheOEt	B-Alanine amide	(0.5 M)	9.0	PheBAlaNH$_2$	3

[a]) 20 µM, 1 mM EDTA

Example 13

Carboxypeptidase Y [a]) catalyzed synthesis of L,L and D,L dipeptides with hydroxyalkylesters of L- and D-Tyrosine and L-Phenylalanine (50 mM) as substrate components and free L-Methionine (0.3 M) as nucleophile in water/ethylene glycol mixtures at pH 9.0

Substrate	% glycol (vol/vol)	Product	Yield %
TyrOEtOH	0	TyrMetOH	10
TyrOEtOH	40	TyrMetOH	8
TyrOEtOH	60[b])	TyrMetOH	5
tyrOEtOH	0	tyrMetOH	56
PheOEtOH	0	PheMetOH	16

[a]) 5 µM, 1 mM EDTA
[b]) 10 µM, 1 mM EDTA

Example 14

Synthesis of L,L-dipeptides catalyzed by carboxypeptidases from barley and wheat at 50 mM initial L-substrate ester concentration at pH 8.0 in water and L-amino-acid and amides as nucleophile components

Enzyme	Substrate	Nucleophile	(conc.)	Product	Yield%
CPD-MII[a])	PheOEt	Methionine	(0.4 M)	PheMetOH	10
CPD-W[b])	PheOEt	Arginine	(0.8 M)	PheArgOH	8

[a]) 20 µM, 1 mM EDTA, 2 M NaCl
[b]) 10 µM, 1 mM EDTA

Example 15

Synthesis of D,L-dipeptides catalyzed by carboxypeptidases from barley and wheat at 50 mM initial D-Phenylalanine ethylester concentration at pH 8.0 in water and free L-amino-acids as nucleophile components

Enzyme	Substrate	Nucleophile	(conc.)	Product	Yield%
CPD-MII[a])	pheOEt	Methionine	(0.4 M)	pheMetOH	15
CPD-W[b])	pheOEt	Arginine	(0.8 M)	pheArgOH	13
CPD-W[b])	pheOEt	Methionine	(0.4 M)	pheMetOH	40

[a]) 20 µM, 1 mM EDTA, 2 M NaCl

[b]) 10 µM, 1 mM EDTA, 2 M NaCl, Reaction time > 20 hr, conversion less than 50 per cent

Example 16

Preparative synthesis of L,L-tyrosylcysteine, TyrCysOH

Procedure

L-tyrosine ethyl ester hydrochloride (1.5 g, 6 mmol) and L-cysteine (15.2 g, 125 mmol) were dissolved in 110 ml of-0,1 M KCl solution containing 1 mM EDTA. pH was adjusted to 8.0 with triethylamine. The reaction was initiated by addition of 7.5 ml of carboxypeptidase Y-solution (16 mg/ml), and was kept at pH 8.0 for the duration of the reaction by the addition of triethylamine under continuous stirring at room temperature. The remainder of the substrate (13.5 g, 54 mmol) was added in portions of 1,5 g during one hour. After 0,5 hour tyrosine precipitation started, and after 3.5 hours the reaction was stopped by heating to 45°C for 20 minutes.

The formed tyrosine was filtered off, and the filtrate was purified by R-preparative HPLC (Waters Prep. L C/system 500A) using two columns (5.7 x 30 cm) packed with 60 um C_{18}-particles and 50 mM acetic acid as eluent. Collected fractions containing pure product were evaporated to dryness in vacuo under repeated additions of absolute ethanol. The remnant was stirred with diethyl ether. Following this, 3.88 g of L,L-tyrosine cysteine (14 mmol, 22%) was isolated by filtration and drying.

Identification

Choride and acetate were not detected, the product being present as a zwitter-ion.

Amino-acid analysis following acid hydrolysis and derivation of Cys by acrylonitrile gave the result:

Try (1.00)

Cys (1.08)

Purity

Following derivation of the cysteine sidechain with acrylonitrile, only one spot was detected on TLC on Kiselgel 60-F using ninhydrine detection (Rf 0.73, eluent : ethyl acetate, butanol, acetic acid and water (1:1:1:1)).

HPLC-purity: 99.5% (nucleosile 7 C_{18}, 0.1 M ammonium phosphate, pH 3.0/acetonitrile, 220 nm).

UV-quantization: 98.5% (293 nm, tyrosine absorbance in 0,1 M NaOH).

Example 17

Preparative synthesis of L,L-methionyl-methionine, MetMet-OH

Procedure

L-methionine ethyl ester hydrochloride (24.6 g, 115 mmol) and L-methionine (20.6 g, 138 mmol) were dissolved in 190 ml of 0.1 M KCl solution containing 1 mM EDTA. pH was adjusted to 9.0 with sodium hydroxide solution, and the reaction was initiated by addition of 14.2 ml of carboxypeptidase Y solution (20 mg/ml). The reaction was stirred overnight at room temperature, and pH was kept at 9.0 through the addition of sodium hydroxide solution by a pH-stat. pH was adjusted to 3.0 with HCl-solution at the end of reaction.

Precipitated methionine was filtered off, and the filtrate was purified by R-preparative HPLC as described in example 16. Collected fractions containing pure product were concentrated by evaporation and finally freeze-dried. This procedure gave 10.6 g (37.8 mmol, 33%) of L-methionyl-L-methionine as a white amorphous powder.

Indentification

No chloride was detected, and only 1.9% (w/w) of acetate was determined, the product being predominantly in zwitter-ionic form. Amino-acid analysis showed methionine following acid hydrolysis and the absence of free methionine in unhydrolysed samples.

Purity

Purity by HPLC: 99.8% (nucleosile 7 C_{18}, 0.1 M ammonium phosphate, pH 3.0/acetonitrile, 220 nm).
Quantization by reaction with trinitrobenzenesulfonic acid (TNBS) and UV-detection: 92.5%.
Water content by Karl Fisher: 1.5%.

Example 18

Preparative synthesis of L,L-tyrosylvalineamide, TyrValNH$_2$

Procedure

L-tyrosine ethyl ester hydrochloride (16.0 g, 65 mmol) and L-valine amide hydrochloride (60 g, 390 mmol) were dissolved in 1150 ml of water and 65 ml of 20 mM EDTA was added. pH was adjusted to 9.0 with sodium hydroxide solution and the reaction was initiated by addition of 12 ml of a carboxypeptidase Y solution (20 mg/ml). The reaction was stirred at room temperature for four hours, and pH was maintained at 9.0 by the addition of sodium hydroxide solution. Following complete conversion the reaction was stopped by raising pH to 11.

The denatured enzyme was filtered off, and the reaction mixture was diluted and purified by successive anion exchange on a Dowex A61x4 column and cation exchange on a Dowex A650Wx4 column using soidum and ammonium acetate salt gradients, respectively, and was finally desalted. Product fractions were combined and evaporated to dryness in vacuo under repeated additions of absolute ethanol, giving 11.2 g L,L-tyrosylvaline amide (40 mmol, 62%) as a white powder.

Identification

1.8% (w/w) of acetate was determined, the product being predominantly in zwitter-ionic form.
Amino-acid analysis following acid hydrolysis gave the following results:
Try (1.01)
Val (0.99)

Purity

HPLC-purity: 99.5% (Novapak C_{18}, 0.1 M ammonium phosphate containing alkylsulfonate, pH 4.5/acetonitrile, 220 nm).
UV-quantization: 97.8% (293 nm, tyrosine absorbance in 0.1 M NaOH).

Example 19

Preparative synthesis of D,L-tyrosyl-arginine, D,L-tyrArg-OH

Procedure

105 g of L-arginine (105 g, 603 mmol) was dissolved in 400 ml of water, and pH was adjusted to 9.0 with HCl-solution. D-tyrosine ethyl ester hydrochloride (6.1 g, 25 mmol) and 5 ml of 0.1 M EDTA were added, and pH readjusted to 9.0. The reaction was initiated by addition of 7 ml of a carboxypeptidase Y solution (20 mg/ml) and pH was kept at 9.0 by addition of NaOH-solution. After 4 hours the reaction was stopped by adjusting pH to 3 with HCl-solution.

The reaction mixture was diluted and purified by cation exchange on a Dowex A650Wx4 column using an ammonium acetate salt gradient, and finally desalted. Collected product fractions were concentrated by evaporation and finally freeze-dried, giving 6.45 g of D,L-tyrosyl-arginine (19 mmol, 77%) as a white powder.

Identification

Acetate and chloride could not be detected, the product being present as a zwitter-ion.

Amino-acid analysis following acid hydrolysis gave the following result:

Arg (1.03)

Tyr (0.87)

Purity

HPLC-purity: 99.5% (Novapak C_{18}, 0.1 M ammonium phosphate with alkylsulfonic acid, pH 4.5/acetonitrile, 220 nm).

Water content by Karl Fisher: 3.4%.

Example 20

Preparative synthesis of D,L-phenylalanylmethionine, D,L-pheMetOH by the use of immobilized carboxypeptidase Y

Immobilization procedure

Carboxypeptidase Y was immobilized on Eupergit C following the recommended procedure of the manufacturer. The immobilization was carried out in the phosphate buffer at pH 7.5, and residual active gel-groups were blocked with ethanolamine at pH 8.0 with subsequent washing.

93% of the enzyme was bound to the gel containing 2.5 mg protein/ml. The Eupergit C coupled enzyme was stored in 10 mM PIPES, 1 mM EDTA, 0.05% hydroxybenzoic acid ethyl ester, pH 7.0 at 4°C.

Synthetic procedure

D-phenylalanine ethyl ester hydrochloride (5.7 g, 25 mmol) and L-methionine (29.8, 200 mmol) were dissolved in 400 ml of H_2O, 5 ml of 0.1 N EDTA was added, and pH adjusted to 9.0 with sodium hydroxide solution, giving a reaction volume of 500 ml. The reaction mixture was continuously stirred and pH kept constant at 9.0 with sodium hydroxide solution, while the solution was being circulated over a column of immobilized CPD-Y at a flowrate of 3 ml/min. The coloumn contained immobilized CPD-Y on Eupergit C prepared as described above and was 2.5 cm x 5.5 cm with a volume of 27 ml, containing a total of 67 mg of CPD-Y. Circulation was continued for 10 hours. pH was then adjusted to 7 with HCl-solution and the product was purified on R-preparative HPLC as described in Example 16.

Collected fractions containing pure product were combined, evaporated in vacuo and finally freeze-dried to give 3.5 g (12 mmol, 48%) of D,L-phenylalanylMethionine as a white, amorphous powder.

Stability of the enzyme preparation

The experiment could be repeated with the same enzyme gel preparation several times without noticable loss in conversion rate and comparable results. Thus, the enzyme is fairly stable at the reaction conditions, further improving process economy.

Indentity of the product

The product was free of chloride, but contained 7.0% (w/w) of acetate and so was partially on acetate form.

Amino-acid analysis showed the absence of free amino-acids, and following acid hydrolysis a ratio of

Met (0.98)

Phe (1.03)

Specific optical rotation using sodium D-line at 25°C and c=0.25 in water was -128.9°

Purity of product

HPLC: 99.6% (nucleosile 7 C_{18}, 0.1 M ammonium phosphate, pH 3/acetonitrile, 220 nm).

Water content by Karl Fisher: 2.8%

Example 21

Preparative synthesis of D,D-phenylalanylphenylalanine ethyl ester hydrochloride, D,D-phepheOEt.HCl

Procedure

D-phenylalanine ethyl ester hydrochloride (2.5 g, 11 mmol) was dissolved in 45 ml of water, and 0.5 ml of 0.1 N EDTA was added. pH was adjusted to 9.0 with sodium hydroxide solution, the substrate being present as a partially oily suspension at the beginning. The reaction was initiated by addition of 3.4 ml of a carboxypeptidase Y solution (20 mg/ml) and was stirred for 2.5 hours at room temperature, pH being kept at 9.0 with sodium hydroxide solution. The reaction was stopped by adjusting pH to 3 with HCl-solution.

The reaction mixture was filtered and purified by R-preparative HPLC as described in example 16.

Collected product fractions were concentrated by evaporation in vacuo and freeze-dried with added HCl-solution, giving 0.49 g (1.3 mmol, 12%) as a white, amorphous powder.

Identification

The product contained 9.2% (w/w) of chloride and no acetate, the product being present as hydrochloride.

Amino-acid analysis showed phenylalanine following acid hydrolysis and no phenylalanine prior to acid hydrolysis.

The product could be further hydrolysed with base, yielding a product chromatographically different from D,L-PhePheOH, and the product itself co-eluted with an L,L-PhePheOEt-standard in the used HPLC-system.

Finally, specific optical rotation using sodium D-line at 25°C and c = 0.5 in acetic acid was found to be -42.7°. This compared with a pure standard of L,L-PhePheOEt, which gave +52.1° under similar conditions. In this case, the discrepancy is believed to be due to the lesser purity of the synthesized peptide.

Purity

HPLC-purity: 82.3% (nucleosile 7 C_{18}, 0.1 M ammonium phosphate, pH 3/acetonitrile, 220 nm).

The impurities detected were chromatographically different from substrate, substrate hydrolysis, product hydrolysis or diastereomers thereof.

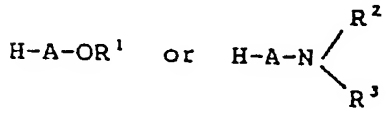
Water content by Karl Fisher: 7.5%.

Claims

1. A process for producing dipeptides having the general formula

H-A-B-Y

wherein A represents an optionally side-chain protected L- or D-α-amino-acid or ω-amino-acid and B represents an optionally side-chain protected L- or D-α-amino-carboxylic acid which may be the same as or different from A, an L- or D-aminophosphonic acid or L- or D-aminosulfonic acid or the corresponding ω-amino-acids or salts and hydrates thereof, and Y is OH or a C-terminal blocking group, characterized by reacting a substrate component, which is an amino-acid derivative having the formula

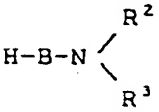


wherein A is as defined above, R^1 represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents or an α-des-amino fragment of an amino-acid, and R^2 and R^3 are the same or different and each represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents, with a nucleophile component which, when A = B may be formed in situ, and is selected from

(a) L-amino-acids having a formula

H-B-OH

(b) L-amino-acid amides having the formula



wherein B is an L-amino-acid, and R^2 and R^3 have the above meaning, except that when R^2 represents

hydrogen, R^3 may also represent hydroxy or amino
(c) L-amino-acid esters having the formula
$H\text{-}B\text{-}OR^4$
wherein B is an L-amino-acid, and R^4 represents alkyl, aryl or aralkyl, and
(d) optionally acid group-protected straight chain or branched aminophosphonic acids or aminosulfonic acids having the formula
$NH_2C_xH_zPO_3H_2$ or $NH_2C_xH_zSO_3H$
wherein x is 1-6 and z is 2-12,
in the presence of a serine or thiol carboxypeptidase from yeast or of animal, vegetable or other microbial origin, in an aqueous solution or suspension having a pH value between 5 and 10.5 optionally containing an organic solvent and/or a salt, and then, if desired, cleaving a present side-chain-protecting group or protective group Y and/or, if desired, converting the resulting dipeptide derivative to a salt or hydrate.

2. A process according to claim 1, **characterized** by using carboxypeptidase Y from yeast as the carboxypeptidase.

3. A process according to claim 2, **character ized** in that the carboxypeptidase used has been purified by affinity chromatography over an affinity resin consisting of a polymeric resin skeleton with a plurality of coupled benzyl succinyl groups.

4. A process according to claim 1, **characterized** in that the carboxypeptidase used is penicillocarboxypeptidase S-1 or S-2 from Penicillium janthinellum, a carboxypeptidase from Aspergillus saitoi or Aspergillus oryzae, a carboxypeptidase C from orange leaves or orange peels, carboxypeptidase C_N from Citrus natsudaidai Hayata, phaseoline from bean leaves or a carboxypeptidase from sprouted barley, malt, sprouted cotton plants, tomatoes, watermelons or Bromelain (pineapple) powder.

5. A process according to claims 1-4, **characterized** in that the carboxypeptidase used has been chemically modified or is a biosynthetic mutant of a natural form.

6. A process according to any of the preceding claims, **characterized** in that the carboxypeptidase enzyme used has been immobilized.

7. A process according to any of the preceding claims, **characterized** by using an aqueous reaction solution or reaction dispersion containing from 0 to 70% of an organic solvent.

8. A process according to claim 7, **characterized** in that the organic solvent is selected from alkanols, dimethyl sulfoxide, dimethylformamide, dimethoxyethane, ethylene glycol or ethyl acetate.

9. A process according to any of the preceding claims, **characterized** by using as substrate component a D- or L-amino-acid ester selected from benzyl esters or straight or branched C_1-C_6 alkyl esters optionally substituted with inert substituents.

10. A process according to any of the preceding claims, **characterized** by using as nucleophile component an amino-acid or amino-acid amide amide having the formula
$H\text{-}B\text{-}OH$ or $H\text{-}B\text{-}NHR^3$
wherein R^3 is hydrogen or C_1-C_3 alkyl and B is an L-amino-acid residue.

11. A process according to claim 1, **characterized** by using as nucleophile component an ester having the formula
$H\text{-}B\text{-}OR^4$
wherein B is an aminocarboxylic acid residue and R^4 is C_1-C_3 alkyl.

12. A process according to any of the preceding claims, **characterized** in that the resulting dipeptide includes one or more C-terminal protective groups Y, and that the group or groups are cleaved enzymatically, preferably by means of the same carboxypeptidase enzyme as was used in the preceding reaction.

13. A process according to any of the preceding claims, **characterized** in that the resulting dipeptide includes one or more side-chain protective groups and that the group or groups are cleaved enzymatically, preferably by means of an esterase or lipase or proteolytic enzyme.

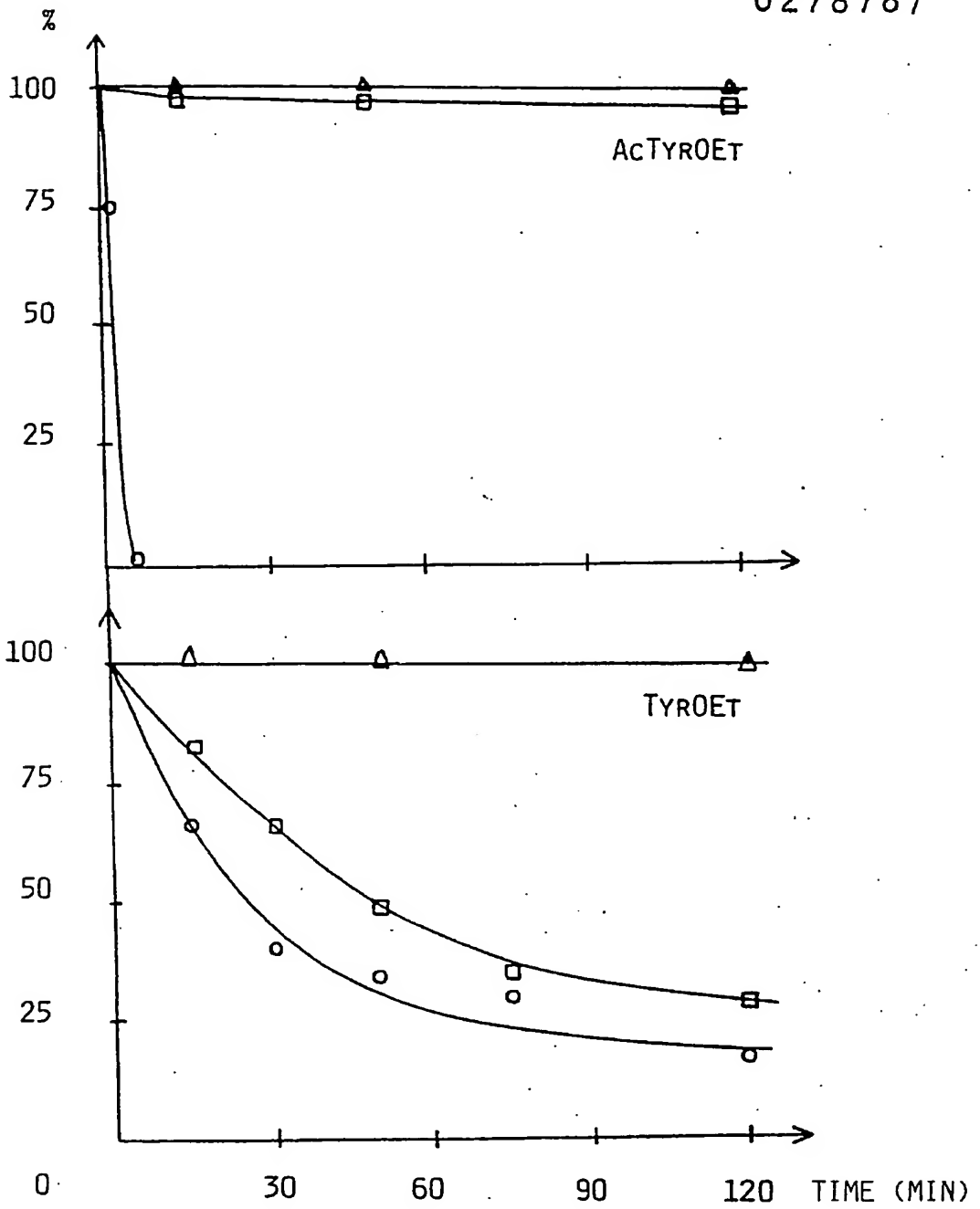


FIGURE 1

Carboxypeptidase Y (10 µM) Catalyzed Hydrolysis of L- and D-TyrOEt and of L- and D-AcTyrOEt (50 mM), 20% DMSO, pH 9,0, 1 mM EDTA

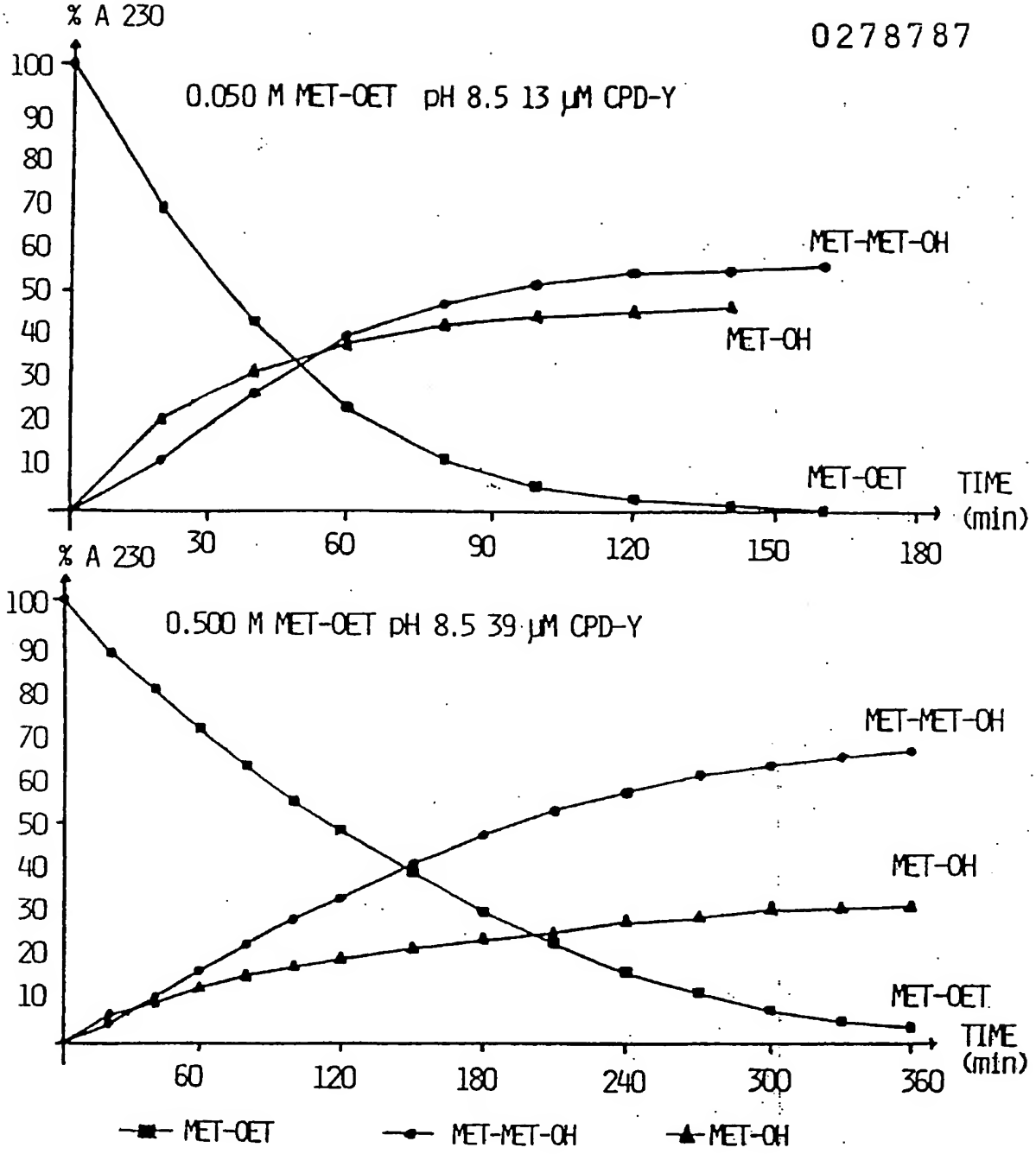


FIGURE 2

Carboxypeptidase Y catalyzed formation of the homodipeptide L,L-Methionyl-Methionine from the single starting compound L-Methionine ethylester at 0.2 M and 0.5 M initial concentration, the nucleophile methionine being generated in situ. No oligomerisation is observed.

% A 230 are per cent arbitrary absorbance units at 230 nm with HPLC-detection.

European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 88 30 1230.4

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	EP-A1-0 074 095 (TOYO SODA MANUFACTURING CO LTD) 16 March 1983 *See page 17, lines 13-35*	1-13	C 12 P 21/00, C 12 P 21/02, C 07 K 1/00
Y	EP-A1-0 086 053 (UNIFIKA LTD) 17 August 1983 *See the claims*	1-12	
Y	EP-A1-0 099 585 (TOYO SODA MANUFACTURING CO LTD) 1 February 1984 *See the claims*	1-12	
Y	EP-A1-0 017 485 (DE FORENEDE BRYGGERIER A/S) 15 October 1980 *See page 5, lines 1-5, page 6-7 and the claims*	1-12	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 P C 07 K
A	US-A-3 972 773 (SAGAMI CHEMICAL RESEARCH CENTER)	1-12	
A	US-A-4 086 136 (SAGAMI CHEMICAL RESEARCH CENTER)	1-12	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
STOCKHOLM	15-04-1988	SIÖSTEEN Y.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
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